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Rapamycin Inhibits Aldolase A Expression During Human Lymphocyte Activation

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Abstract Rapamycin (RAPA) strongly inhibits lymphocyte activation and proliferation, but does not affect most of the activation-related gene expression at the mRNA level. In order to understand the mechanism of action of RAPA and to gain further insights in lymphocyte signalling which is impaired by RAPA, we screened for RAPA-sensitive genes using differential hybridization. The expression of human aldolase A gene was found to be inducible during T and B cell activation, and the induction was repressed by RAPA at both the mRNA and enzymatic levels. The other two important immunosuppressants, cyclosporin A and FK506, also inhibited the mitogen-induced upregulation. However, none of these three drugs inhibited the constitutive expression. There was no fluctuation of aldolase A expression during the cell cycle, and RAPA failed to block the first cell cycle after synchronization in Jurkat cells. However, the second cycle was hampered by RAPA, and this was correlated with the inhibition of aldolase A expression during this later stage. Since aldolase A is a key enzyme in glycolysis and lymphocytes mainly depend on glycolysis for energy supply, the data from this study suggest that aldolase A might be one of the downstream targets of RAPA. The inhibition of the enzyme upregulation might deprive the cells of additional supply of energy, and prevent the cells from entering an optimal status for proliferation.

Key words: lymphocyte activation, Krebs cycle, energy metabolism, immunosuppressives, cell cycle

Rapamycin (RAPA), a natural product from *Streptomyces hygroscopicus*, is a potent immunosuppressive drug [Vezina et al., 1975; Sehgal et al., 1975; Calne et al., 1989]. It strongly inhibits lymphocyte activation and proliferation, while it has low toxicity to resting cells [Dumont et al., 1990; Luo et al., 1992]. RAPA's possible clinical application in organ transplantation and in autoimmune diseases is obvious, and the clinical trials are currently in progress. Cell biologists also have a kin interest in this drug. The rationale is simple: since the cell proliferation is very sensitive to RAPA, the target of RAPA in the cell must have a vital function, and must occupy a critical position in the signal transduction pathway from the cell membrane to nucleus.

In the past several years, many groups of scientists including us have been searching for the direct or indirect cellular targets of RAPA. We now know that RAPA forms a complex with a 12 KD cytoplasmic FK506-binding protein (FKBP12) [Harding et al., 1989; Siekierka et al., 1989]. The RAPA-FKBP complex will then bind to putative cytoplasmic target proteins, which is termed TOR1 and TOR2 (target of rapamycin) in yeast [Kunz et al., 1993; Helliwell et al., 1994], and FRAP and RAFT1 (rapamycin and FK506 targets) in mammalian cells [Brown et al., 1994; Sabatini et al., 1994]. FRAP and RAFT have a high degree of homology to the yeast TOR gene product. The C-terminal amino acid sequences of TOR, FRAP, and RAFT also have homology with the catalytic domain of both PI-3 kinase and PI-4 kinase. Trace amount of PI-4 kinase activity of RAFT1 and TOR2 has been demonstrated, although the RAPA-FKBP12 complex has no significant inhibitory effect on the activity [Sabatini et al., 1995; Cardenas and Heitman, 1995]. A most recent study has shown that in yeast TOR2 is membrane associated and localizes to the surface of the yeast vacuole, and

Abbreviations used: CsA, cyclosporin A; PHA, phytohemagglutinin; RAPA, rapamycin.

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is involved in vacuolar morphology and segregation [Cardenas and Heitman, 1995]. Obviously, the function of TOR and RAFT needs further elucidation, and so does FKBP.

Different isoforms of FKBP have been found in various locations in the cells, and consequently they might have diverse functions. The ubiquitous FKBP12 can form a complex with the 565 KD ryanodine receptor, four of which form intracellular Ca^{++} release channel of sarcoplasmic and endoplasmic reticula [Brillantes et al., 1994]. A 25 KD FKBP is found in the nuclei and mitochondria [Breiman et al., 1992; Jin and Burakoff, 1993], and could form a complex with case in kinase II. A 13 KD FKBP has a potential endoplasmic reticulum retention sequence, and is found in the heavy membrane fraction [Jin et al., 1991]. A 52 KD isoform of FKBP is also called heat shock protein 56 (HSP56), and is inducible during stress. It associates with the 90 KD HSP and is a component of the steroid receptor complex [Peattie et al., 1992]. The FKBP52 is localized both to microtubules in the cytoplasm and to the nucleus [Czar et al., 1994]. These findings raise the question whether the FKBP12 and its association with TOR or FRAP/RAF1 represent the only relevant and sufficient mechanism for RAPA's effect.

Several molecules in the signalling pathway are affected by RAPA, and they are probably downstream of the primary target. Activation of p70 S6 ribosomal kinase is repressed by RAPA [Kuo et al., 1992; Chung et al., 1992; Price et al., 1992], and there is evidence that the activation is controlled by the kinase activity of RAFT [Brown et al., 1995]. Whether the inhibition of p70 S6 kinase is responsible for the major drug effect is questionable, because in an erythroid cell line MEL, the kinase activity can be totally inhibited by RAPA while the cells still proliferate vigorously [Calvo et al., 1994].

Since the p70 S6 kinase is likely involved in protein synthesis, several groups have followed this clue and examined the effect of RAPA on protein translation. It has been reported that during T cell activation, RAPA preferentially inhibits up-regulation of ribosome mRNA and prevents synthesis and accumulation of ribosomal proteins [Terada et al., 1994, 1995]. However, it is yet to be understood why general protein synthesis is only mildly affected by RAPA. It has also been found that phosphorylation of PHAS-1, which is a nonphosphorylated form of an inhibitor of the mRNA cap binding protein eIF (eukaryrotic initiation factor), is sensitive to RAPA [Graves et al., 1995].

Some molecules controlling the cell cycle are also affected by RAPA. The drug represses interleukin 2-dependent activation of $p33^{CDK2}$ and $p34^{CDC2}$ kinases in T lymphocytes [Morice et al., 1993]. A further study following this direction shows that RAPA prevents an inhibitory protein $p27^{kip1}$ to dissociate from the cyclin E/CDK2 complex during IL-2-induced T cell proliferation, and thus prevents the cells from leaving the quiescent status [Nourse et al., 1994]. Whether the $p27^{kip1}$-mediated mechanism can be generalized to non-IL-2 driven cell proliferation is to be investigated.

RAPA's effect on the expression of some transcriptional regulators and some cellular proteins has been reported. A protooncogene c-jun upregulation during lymphocyte activation [Shan et al., 1993], the expression of CCAAT/enhancer binding protein α during 3T3-L1 cell differentiation [Yeh et al., 1995], and the augmentation of human NADP-dependent isocitrate dehydrogenase expression after mitogen-stimulation in lymphocytes [Luo et al., 1996], are inhibitable by RAPA. However, the mRNA expression of most genes so far studied including many cytokines, whether they are constitutively expressed or induced after stimulation, are not sensitive to RAPA treatment [Tocci et al., 1989; Shan et al., 1994].

In brief, we are not totally certain about RAPA's primary target(s). Further, we lack a good understanding of the interaction of the known secondary targets. We reasoned that, if we could find additional secondary targets of RAPA, we would be in a better position to fill up some gaps and establish some links among the known components of the signalling pathway.

Since RAPA rarely inhibits gene expression at the mRNA level, the genes which are sensitive to RAPA might have a good probability of being secondary targets of RAPA. In this study, we used differential hybridization to identify such RAPA sensitive genes. Human aldolase A was found to be inducible during lymphocyte activation, and the induction of the mRNA expression and the enzymatic activity were repressed by RAPA. The implication of such a finding is discussed.

MATERIALS AND METHODS

Reagents

Lymphoprep was purchased from NYCOMED (Oslo, Norway), Human recombinant IL-2 and IL-4 from Genzyme (Boston, MA), *Staphylococcus aureus* Cowan I (SAC) from Calbiochem (La Jolla, CA), and PHA from Sigma (St. Louis, MO). RPMI 1640, FCS, penicillin/streptomycin, and L-glutamine were purchased from GIBCO/BRL (Burlington, ON). RAPA was a gift from Wyeth-Ayerst Research (Princerton, NJ), CsA a gift from Sandoz Canada (Laval, Quebec), and FK506 a gift from Fujisawa (Deerfield, IL). The random primer labeling kit was the product of Pharmacia-LKB (Baie D'Urfe, Quebec). The sequenase 2.0 and [α-^{35}S]-dATP were from Amersham (Oakville, ON), and [α-^{32}P]-dCTP (3,000 μCi/mmol) and Biotrans nylon membranes from ICN (Mississauga, ON).

Differential Hybridization

The pcDNA I plasmic cDNA library of activated human B cells was constructed as described previously [Shan et al., 1994]. Eighteen thousand single colonies were innoculated into 96-well cell culture plates, and subsequently transfered onto LB-agar plates in duplicates. The colonies were then lifted onto nylon membranes. The colony hybridization was carried out as detailed by Ausubel et al. [1991]. Two ^{32}P-labeled cDNA probes were prepared for the differential hybridization. One was reverse-transcribed with random primers using template mRNA from PBMC activated by PHA (1.5 μg/ml) for 16 h. The other was reverse-transcribed from mRNA of PBMC activated similarly by PHA for 16 h but in the presence of 80 nM RAPA. The two duplicate sets of membranes were hybridized with these two probes. The intensities of the corresponding colonies in the autoradiograph were compared, and those with apparent differences were selected and subjected to a second round of similar differential hybridization for confirmation.

Cell Culture

PBMC were prepared by lymphoprep gradient as described before [Luo et al., 1993; Shan et al., 1994]. Tonsilar T cells were prepared by one cycle of SRBC rosetting and such preparation contained 90–93% CD3$^+$ cells. The remaining tonsillar cells were referred to as the tonsillar B cells, which were about 90% CD20$^+$ cells. The cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, and antibiotics.

DNA Sequencing

cDNA was sequenced with the chain termination method on double-stranded plasmid DNA using Sequenase 2.0 and [α-^{35}S]-dATP as described previously [Shan et al., 1994].

Northern Blot Analysis

The method is described in our previous publication. Briefly, lymphocyte total RNA was extracted with the guanadine/CsCl method [Shan et al., 1993, 1994]. The RNA was electrophoresed on 0.85% agarose-formaldehyde gel, transfered onto Biotrans nylon membranes, and crosslinked with UV irradiation. A 1.1 Kb HindIII/XbaI fragment of aldolase A cDNA [positions 300 to 1,407 according to Sakakibara et al., 1985] and a 0.9 Kb HindIII/XbaI fragment of H12.3 cDNA [Shan et al., 1994] were labeled with ^{32}P using random primers and were used as probes. The blots were hybridized at 42°C overnight with the probes. The final washes of the membranes were at 56°C for 30 min with 0.1 × SSC containing 0.1% SDS.

Aldolase Assay

Lymphocytes were sonicated in cold PBS. The cell debris was cleared by centrifugation, and the supernatants were assayed for aldolase activity using a kit purchased from Sigma.

Synchronization of Jurkat Cells

Jurkat cells were first cultured for 24 h in isoleucine deficient RPMI 1640 medium supplemented with 10% extensively dialyzed FCS. The cells were then cultured in complete RPMI 1640 medium in the presence of 1 mM hydroxyurea for 16 h. As a consequence, these cells were synchronized and blocked at the G_1/S boundary. The blockage was released by washing out hydroxyurea and culturing the cells in complete medium. The cells were cultured in the absence or presence of RAPA, and were then sampled at different time points after the release for flow cytometry analysis.

RESULTS

Differential Hybridization

In order to identify in lymphocytes genes whose expressions were sensitive to RAPA, a

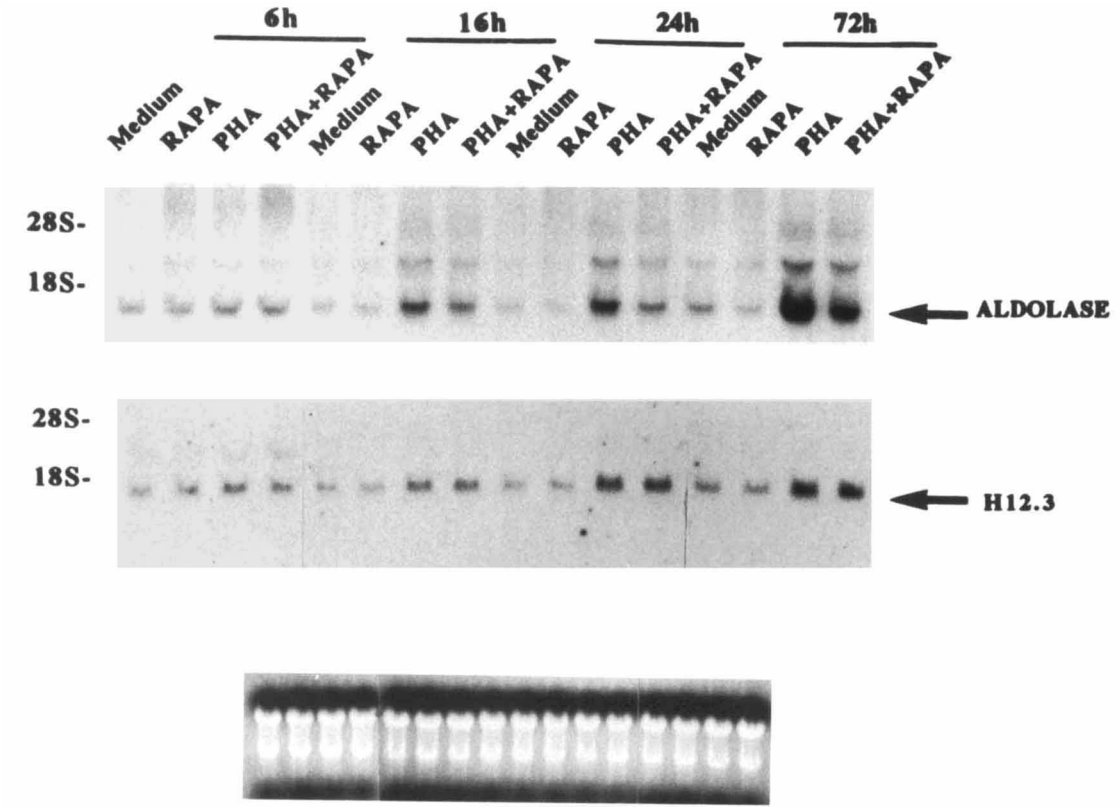

Fig. 1. Aldolase A mRNA expression was induced during T cell activation and the induction was inhibited by RAPA. Human tonsillar T cells were stimulated with PHA (1.5 μg/ml) in the absence or presence of 10 nM RAPA. The cells were harvested at different time points as indicated, and the total RNA was subjected to Northern blot analysis, using ^{32}P-labelled 1.1 Kb aldolase A cDNA as a probe. After the signal decayed, the same membrane was rehybridized with a cDNA probe of the G protein-related gene H12.3, as an internal control of RNA loading. The ethidium bromide staining of the 28S and 18S ribosomal RNA is also presented as another control for RNA loading. The positions of aldolase A and H12.3 message are indicated on the right side.

plasmid cDNA library of activated B cells was screened with differential hybridization, using reverse transcribed probes prepared from mRNA of activated T cells with or without RAPA treatment. After two rounds of differential hybridization, about 200 clones were selected. These clones were subjected to another screening with a pool of mixed probes consisting of Alu repeats, cDNAs of mitochondrial genes, and cDNAs of ribosomal proteins. One hundred and ten clones survived this third round of screening, and these clones were partially sequenced. The cDNAs of 60 of these clones were also used as probes in Northern blot analysis of T cells which were activated by PHA for 16 h in the absence or presence of RAPA. Thirty-four of the cDNA probes could detect discrete bands. Among the 34 corresponding genes, 19 had no activation-related changes of expression and were not sensitive to RAPA. There were 15 genes whose expressions were upregulated after the activation, and in 7 out of the 15 the upregulations were reproducibly inhibited by RAPA. Aldolase A was one of the seven, and its regulation of expression by RAPA was further analyzed in detail.

Aldolase A Expression in T Cells Was Inhibited by Immunosuppressants

Human tonsillar T cells were activated by PHA for different periods of time in the absence or presence of different immunosuppressants. The expression of stable-state aldolase A mRNA was evaluated by Northern blot analysis. Aldolase A mRNA was inducible after 16 h of stimulation, and the upregulated expression lasted until at least 72 h (Fig. 1). RAPA at 10 nM strongly inhibited the upregulation. As an internal control, the same membrane was rehybridized with the cDNA probe of H12.3, which is a G protein β subunit-related gene and is also inducible during lymphocyte activation. In contrast to aldol-

ase A, the H12.3 expression was not sensitive to RAPA.

The effect of CsA and FK506, the other two important immunosuppressants sharing certain similarity with RAPA in their mechanism of action in terms of immunophilin binding, on aldolase A expression was also examined. The kinetic study was carried out up to 40 h after activation, because it was difficult to obtain enough cells from a longer culture. Unlike RAPA, CsA at 1 μM inhibited both aldolase A and H12.3 expression at 40 h (Fig. 2a). FK506 (10 nM), which shares structural similarity to RAPA, also inhibited aldolase A expression (Fig. 2b).

At the protein level, the enzymatic activity of aldolase A increased following PHA stimulation of T cells (Fig. 3). At 40 h, the increase was 1.6-fold on per mg protein basis. Since the total protein per cell also increased about 3-fold during this period [Wang et al., 1980] (our own observations), the increase of the enzymatic activity per cell was about 5-fold. RAPA at 10 nM could inhibit the activity to the preactivation level. FK506 and CsA also caused strong inhibition at 40 h.

IL-2 and IL-4 play important roles in T cell differentiation and proliferation. They could augment mitogen-induced T cell proliferation [Okada et al., 1979; Paul, 1991]. However, these two lymphokines did not further increase PHA-induced aldolase A expression, although they could augment the PHA-induced expression of a G protein β subunit-like gene H12.3 (Fig. 4).

RAPA's Effect on Aldolase A Expression of T Cells Did Not Depend on Cell Cycle But Depended on Duration of Drug Treatment

Since RAPA prevents resting T cells from entering the cell cycle, and the drug also inhibited the upregulation of aldolase A during T cell activation, we next asked whether the aldolase A expression was cell cycle dependent, and whether RAPA could suppress the putative periodic expression of the gene. Human T cell leukemic cell line Jurkat was synchronized by isoleucine starvation plus hydroxyurea blockage. More than 80% of the cells were at G_1/S boundary at the time of hydroxurea release (0 h, Fig. 5a). At 6 and 12 h, the peak shifted to S and G_2/M phase respectively. At 24 h, most of the cells completed one cycle and re-entered the G_1 phase. The second cycle completed at around 48 h with decreased synchronization. In this model, aldolase A showed no periodic fluctuation in different phases of the cell cycle (Fig. 5b). RAPA's effect on aldolase A was not significant in the first cell cycle, and the drug did not block or delay the first cycle. However, the second cycle was delayed by RAPA, and this coincided with the inhibition of aldolase A expression at 40 h.

RAPA Inhibited Aldolase A Expression in B Cells

We have also studied RAPA's effect on aldolase A expression in B cells. The tonsillar T cells were activated by SAC and IL-2, and RAPA (10 nM) or CsA (1 μM) was added in the beginning of the culture. Both RAPA and CsA inhibited aldolase A expression at 16 h, but the inhibition was no longer obvious at 40 h (Fig. 6a). This phenomenon was reproducible. At the enzymatic level, the inhibition was apparent at 40 h but not at 16 h (Fig. 6b). Therefore the kinetics of the inhibition in T and B cells seemed different at mRNA levels, but was similar at the protein level.

DISCUSSION

In order to obtain insights in the mechanism regulating lymphocyte activation and proliferation, we used RAPA as a probe to identify genes sensitive to this potent immunosuppressant. While other strong immunosuppressants such as CsA and FK506 inhibit most inducible genes during the activation, RAPA does not [Tocci et al., 1989; Shan et al., 1994]. Such a feature certainly makes RAPA-sensitive genes better candidates which might be essential and relevant in the activation program. As expected, among a large panel of genes examined, only a few were repressed by RAPA, and aldolase A was among the few.

Aldolase (EC 4.1.2.13) is an enzyme in the glycolysis pathway. It reversibly cleaves fructose-1, 6-diphosphate into two trioses, i.e., dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. This enzyme has three distinct isozymic forms, aldolase A, B, and C [Horecker et al., 1972]. The developing embryo expresses aldolase A [Lebherz and Rutter, 1969]. In adult, aldolase A is found alone in muscle, co-expressed with aldolase B in intestine and kidney, and co-expressed with aldolase C in brain. In lymphocytes, aldolase A is the predominant isozyme [Steinhagen-Thiessen and Hilz, 1979].

Although lymphocytes have all the enzymes for glycolysis and the Krebs cycle, the energy is mainly produced through glycolysis in these cells [MacHaffie and Wang, 1964]. Furthermore, dur-

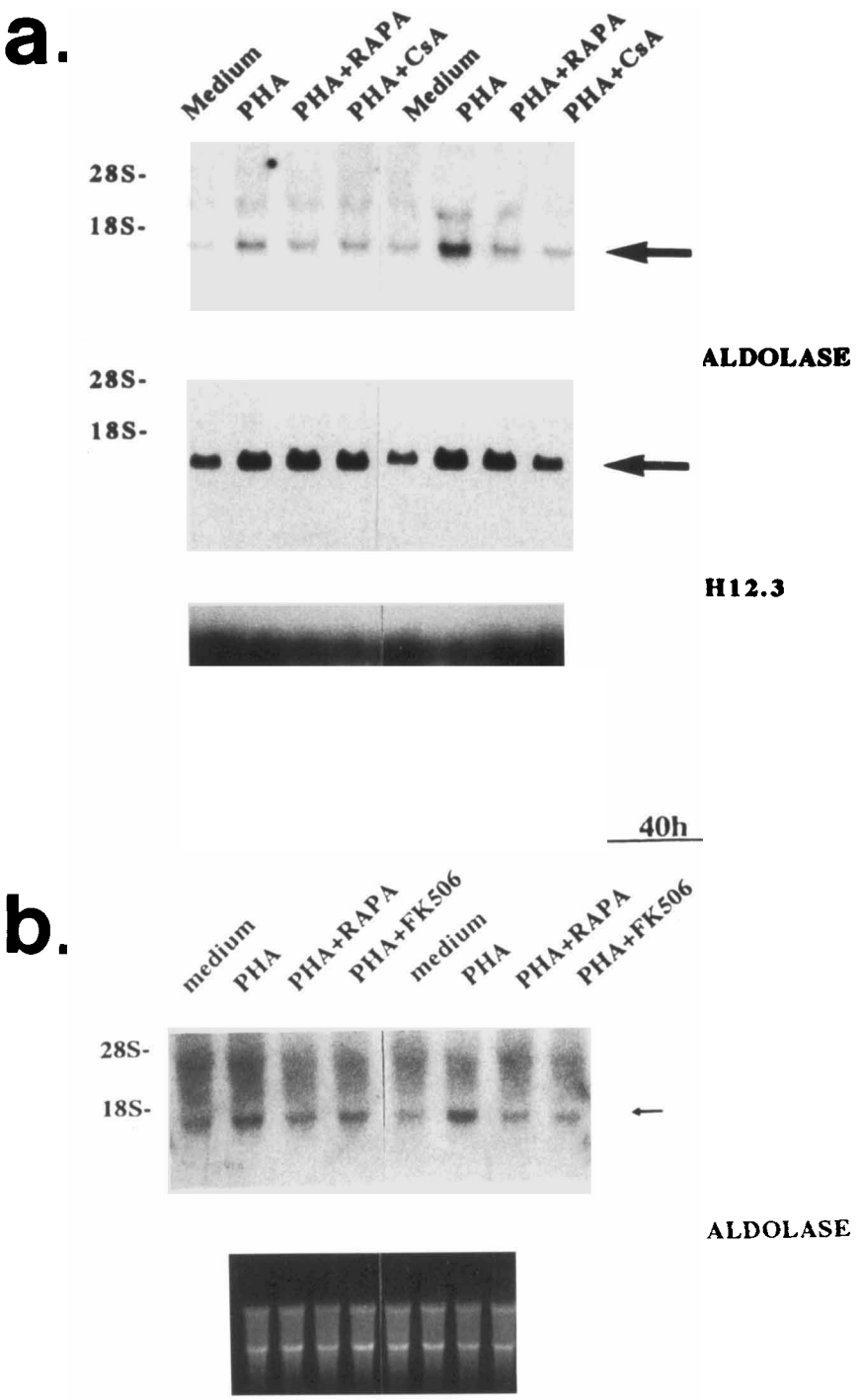

Fig. 2. The induction of aldolase A mRNA expression during T cell activation is sensitive to CsA and FK506. Human tonsillar T cells were stimulated with PHA in the absence or presence of RAPA (10 nM), CsA (1 μM), or FK506 (10 nM). The total RNA of the cultured cells were analyzed by Northern blots. The membrane A was rehybridized with the H12.3 probe as an internal loading control. The ethidium bromide staining of ribosomal RNA is also presented as a loading control. **a:** In the presence of CsA. **b:** In the presence of FK506.

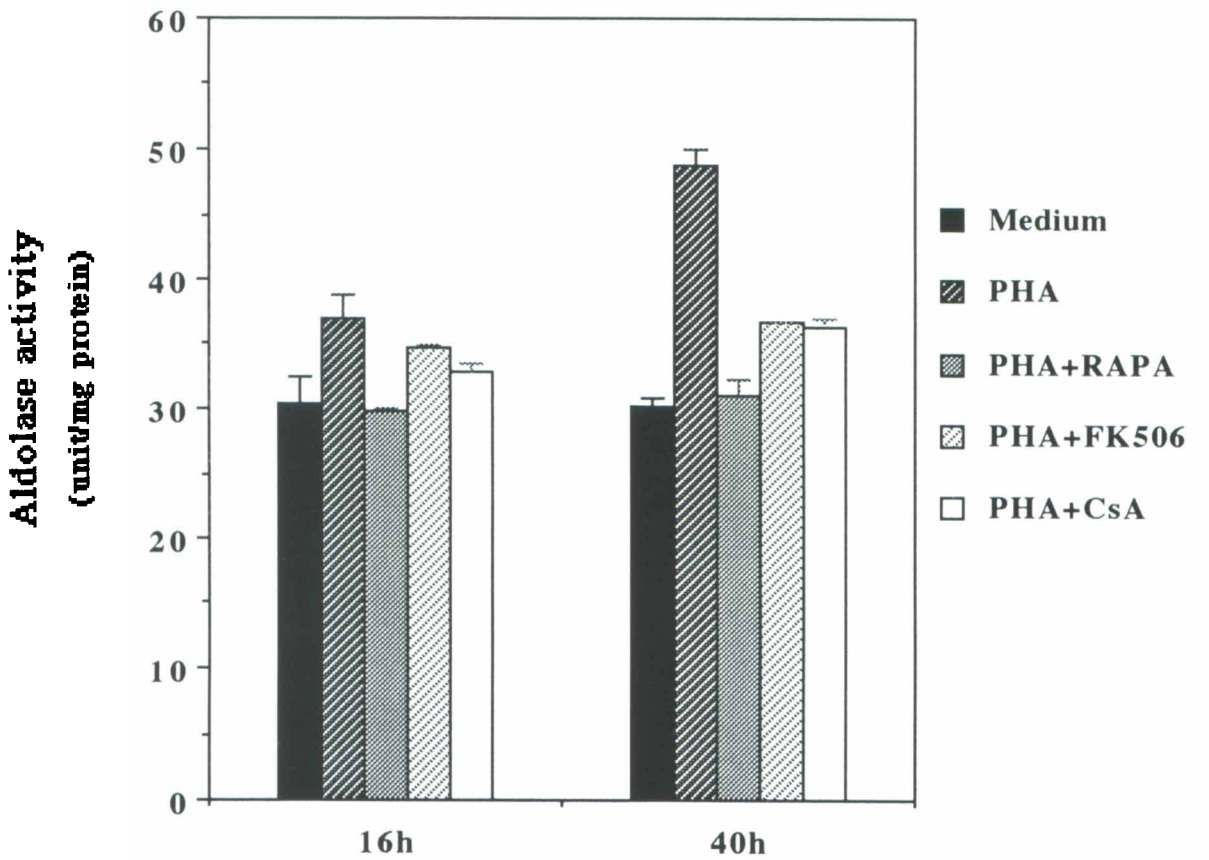

Fig. 3. The effect of immunosuppressants on aldolase A enzymatic activity in T cells. Tonsillar T cells were activated by PHA (1.5 μg/ml) in the presence of RAPA (10 nM), FK506 (10 nM), or CsA (1 μM). The aldolase A activity of the cell lysates was measured at 16 and 40 h. The mean and SD of duplicate samples are presented. One representative result of three similar ones is shown.

ing T cell activation it is known that glycolysis increases about eightfold comparing to unstimulated cells [Roos and Loos, 1970], while the Krebs cycle activity only augments marginally [Roos and Loos, 1973]. The increased glyolysis is probably essential to meet the additional requirements for energy and anabolic intermediates during cell activation and proliferation. Although hexokinase and phosphofructokinase are the rate limiting enzymes in the glycolysis pathway [Wang et al., 1980], restricted supply of aldolase could also hamper the cell function. For example, in human aldolase A deficiency, which is caused by a one base mutation in the aldolase A gene, the enzyme is heat labile and only has 5% of the normal activity. The patients with such a defect manifest hemolytic anemia [Kishi et al., 1987], since red blood cells exclusively depend on glycolysis for energy supply. An aldolase inhibitor vanadate could similarly cause hemolytic anemia in animals [Al-Bayati et al., 1990].

In this study, we have found that the aldolase A expression during T and B cell activation was augmented and the augmentation was repressed by RAPA as well as by two other immunosuppressants CyA and FK506. It is interesting to note that RAPA, CsA, and FK506 mainly inhibited the induced part of aldolase A expression, while it had little effect on the constitutive expression. None of the three immunosuppressants repressed the aldolase A expression below the preactivation level. Therefore, the resting cells should be insensitive to these drugs. Indeed this is the case.

In the synchronized leukemic Jurkat cells, there was no periodic fluctuation of aldolase A expression, although the expression was high throughout different phases. This was probably due to that these cells were already in the proliferation mode, and all the metabolic machinaries including aldolase A were tuned to a high rate. RAPA did not immediately prevent the cells

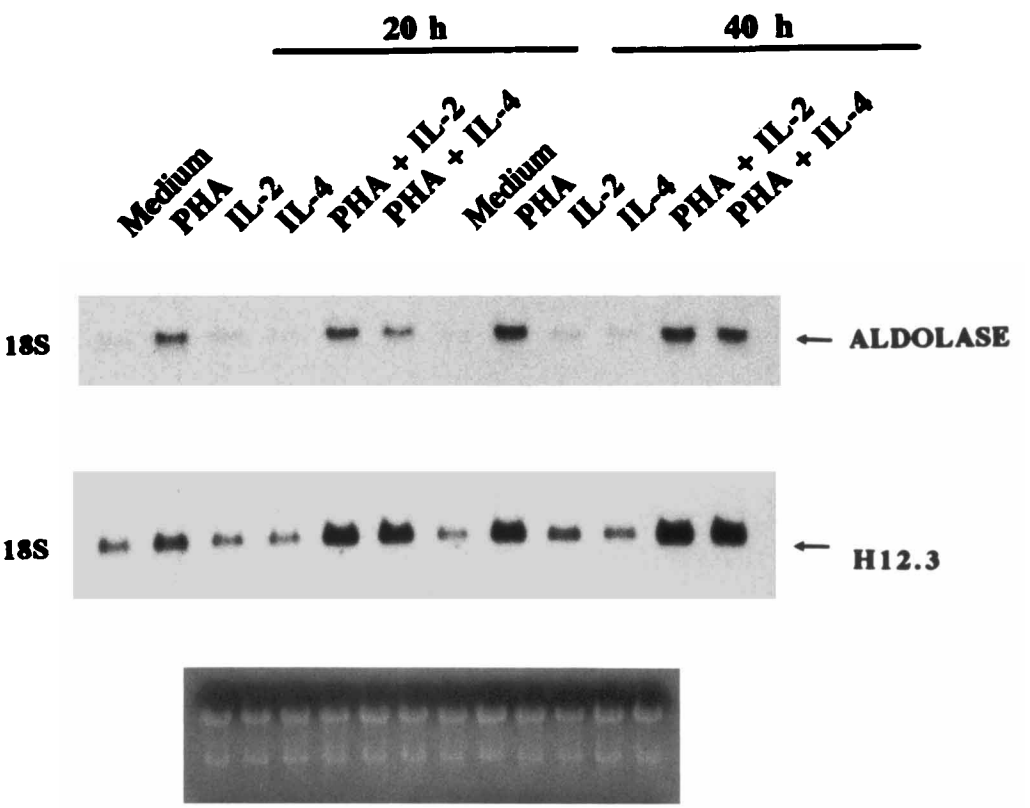

Fig. 4. The effect of IL-2 and IL-4 on PHA-stimulated aldolase A expression in T cells. Tonsillar T cells were stimulated by PHA (1.5 μg/ml) in combination with human recombinant IL-2 (100 μ/ml) or IL-4 (15 ng/ml). The aldolase A mRNA was analyzed by a Northern blot. The same membrane was rehybridized with the H12.3 probe as an internal control. RNA loading is indicated in the bottom panel.

from entering the S phase in the first cycle during the drug treatment. Since aldolase A has a long half life of 118 h, it is likely that these cells still have enough active enzyme to tide them over for one cycle. Besides, the de novo aldolase A expression was not sensitive to RAPA until 20 h. After a longer culture period, RAPA's effect on aldolase A expression became apparent at 40 h. The preexisting enzyme must have also decreased at this time. This was correlated with a partial blockage of the second cycle in RAPA treated cells.

The molecular mechanism of the effect of RAPA on aldolase A expression needs to be investigated. Since the mitogen-induced upregulation of aldolase A mRNA is repressed by RAPA, it is conceivable that the drug inhibits the expression at least partly at the level of transcription. The regulation of human aldolase A gene is complex. Three alternative promoters, pN, pM, and pH are localized within a 1.6-kbp region [Maire et al., 1987]. pN and pH are ubiquitously functional, while pM is skeletal muscle-specific. pN and pH give rise to multiple species of mature mRNA with length variation of about 128 bp, which could be discerned by RNAase H mapping [Gautron et al., 1991]. While pN does not seem to have discrete cis-acting elements [Gautron et al., 1991], a strong enhance region shown to be necessary for both pH and pN activities in cultured cells has been localized upstream of pH [Concordet et al., 1991]. In this region, there are two AP-1 consensus binding sites [Joh et al., 1986], at least one AP-2-like binding site [Imagawa et al., 1987], and one SV40 "core enhancer" for AP-3 [Mitchell et al., 1987]. Since both RAPA and CyA could inhibit the expression of c-jun [Shan et al., 1993], which is the component of AP-1, one possible mechanism of RAPA's effect is by repressing the trans-acting factor AP-1.

Previous study has shown that RAPA could inhibit the association of eukaryotic initiation factor eIF4E and the translational regulator PHAS-1 [Graves et al., 1995], and selectively inhibit translation of certain proteins such as

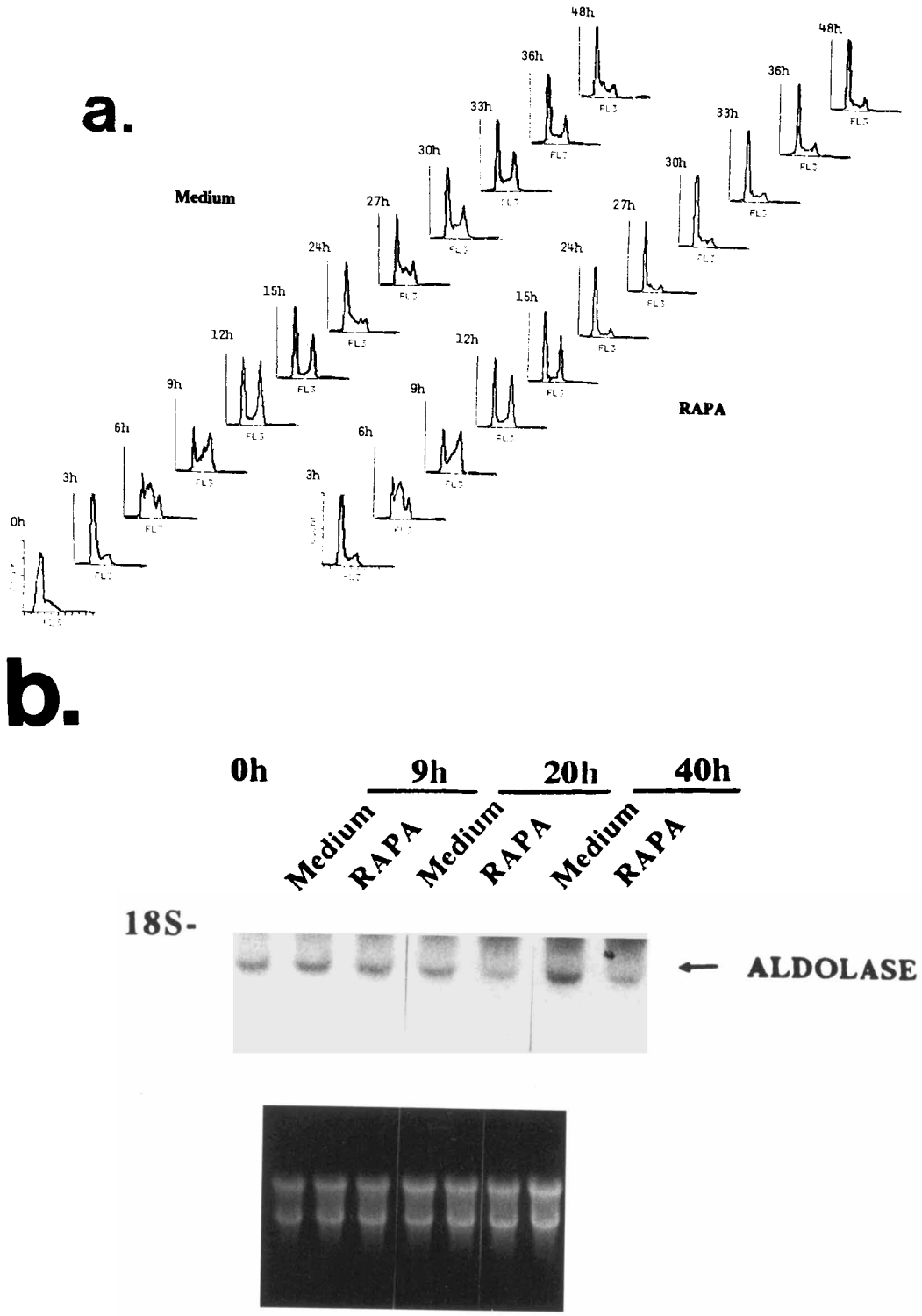

Fig. 5. Effect of RAPA on aldolase A expression in synchronized Jurkat cells. **a:** Cell cycle analysis of synchronized Jurkat cells with or without RAPA treatment. Jurkat cells were blocked at the G_1/s boundary by isoleucine starvation plus hydroxyurea treatment, and were released at 0 h by washing out hydroxyurea. The cells were then cultured in the absence or presence of 100 nM RAPA. The cell cycle of these synchronized cells was monitored by flow cytometry employing propidium iodide staining. The time after the release was indicated. **b:** Northern blot analysis of aldolase A expression in the synchronized Jurkat cells. The cells were cultured in the absence or presence of 100 nM RAPA since their release from hydroxyurea.

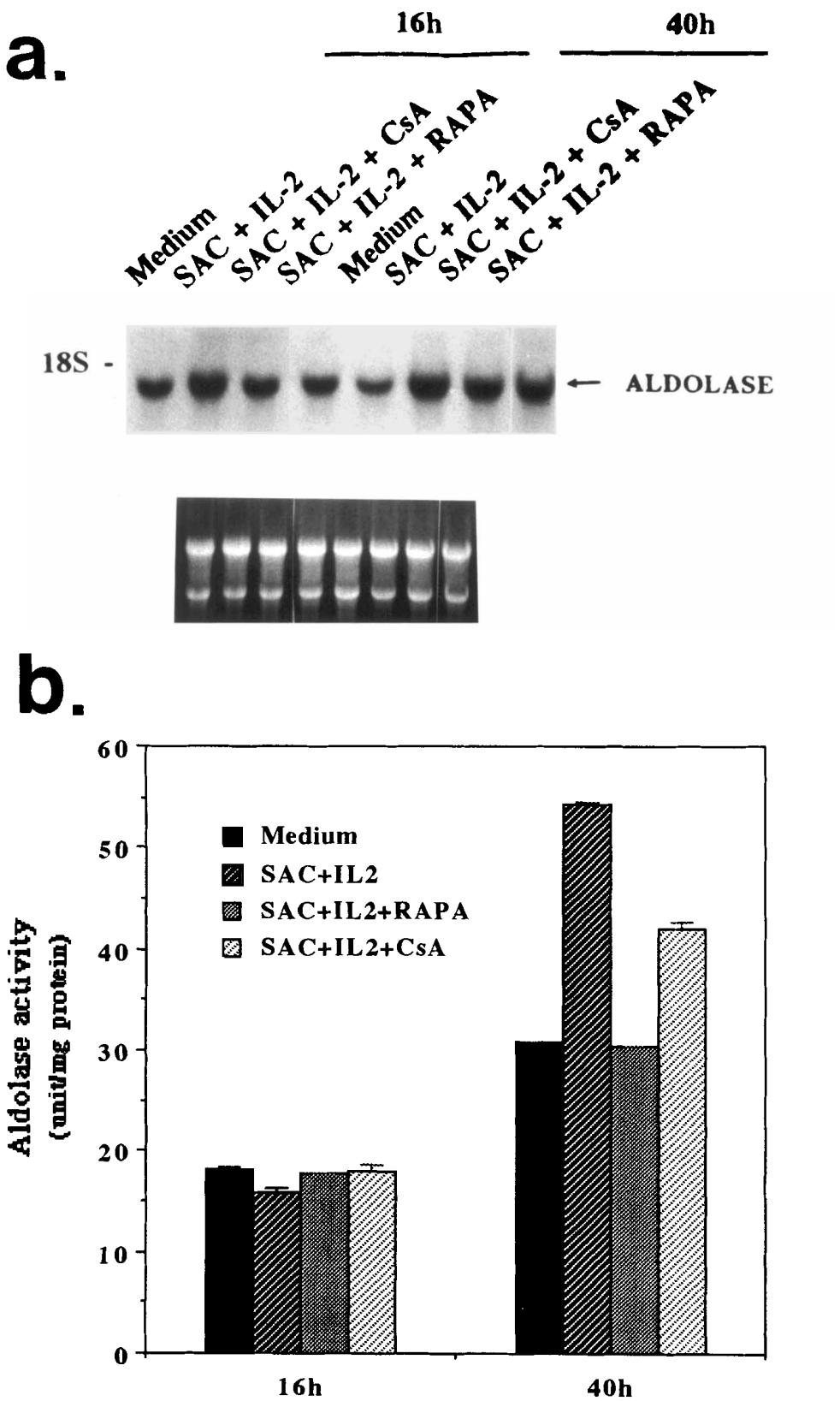

Fig. 6. The effect of RAPA and CsA on aldolase A expression in B cells. Tonsillar B cells were stimulated with SAC (1:10,000 dilution) plus IL-2 (100 μ/ml) in the absence or presence of RAPA (10 nM) or CsA (1 μM). **A:** The aldolase A mRNA expression was analyzed by Northern blot using total RNA. The 28S and 18S bands were stained with ethidium bromide as a control for RNA loading. **B:** The aldolase A enzymatic activity of the cultured cells were assayed 16 and 40 h after the stimulation.

elongation factor eIF-2 and ribosomal proteins [Terada et al., 1994]. Thus, the aldolase A expression could also be affected by RAPA through this route either directly at the translation of its protein, or at the translation of the trans-acting nuclear proteins controlling the aldolase A transcription. These possibilities need to be further examined.

When resting lymphocytes are activated by mitogens, there are early signalling events, some of which, such as activation of the p70 S6 kinase and phosphorylation of PHAS-1, are inhibitable by RAPA. The activation signal will lead to protein and eventually DNA synthesis. Protein synthesis normally starts to increase several hours after the activation, and DNA synthesis 24–48 h later. Several glycolytic enzymes including aldolase A are upregulated quite before the onset of DNA synthesis [Diamond et al., 1978; Matrisian et al., 1985]. We have therefore identified a RAPA-sensitive intermediate step between the onset of protein and DNA synthesis and filled a gap in between. The impact of RAPA on the aldolase A will probably deprive the activated cells from the additional supplies of energy and anabolic elements, and prevent them from further optimal protein and DNA synthesis. This might be one of the mechanisms through which RAPA exerts its inhibitory effect on lymphocyte proliferation.

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